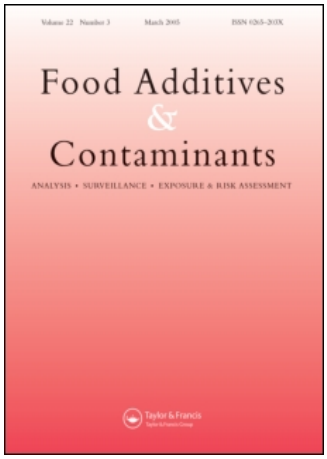

Food Additives & Contaminants
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713599661

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Online Publication Date: 01 October 2007
To cite this Article: Kale, S. P., Cary, J. W., Hollis, N., Wilkinson, J. R., Bhatnagar, D., Yu, J., Cleveland, T. E. and Bennett, J. W. (2007) 'Analysis of aflatoxin regulatory factors in serial transfer-induced non-aflatoxigenic **Aspergillus parasiticus**', Food Additives & Contaminants, 24:10, 1061 - 1069
To link to this article: DOI: 10.1080/02652030701564563
URL: http://dx.doi.org/10.1080/02652030701564563

Food Additives and Contaminants, October 2007; 24(10): 1061–1069

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Analysis of aflatoxin regulatory factors in serial transfer-induced non-aflatoxigenic *Aspergillus parasiticus*

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(*Received 17 May 2007; accepted 10 July 2007*)

Abstract
Aflatoxins (AFs) are carcinogenic secondary metabolites of *Aspergillus parasiticus*. In previous studies, non-toxigenic *A. parasiticus sec*− (for secondary metabolism negative) variants were generated through serial transfer of mycelia from their toxigenic *sec*+ (for secondary metabolism positive) parents for genetic and physiological analysis for understanding regulation of AF biosynthesis. Previous studies have shown no difference in the DNA sequence of *afl*R, a positive regulator of AF production, in the *sec*+ and *sec*− strains. In this study, AflJ, another positive regulator of AF production, *lae*A, a global regulator of secondary metabolism, and the intergenic region between *afl*R and *afl*J, were analysed to determine if they play a role in establishment of the *sec*− phenotype. The study showed that while this sequence identity extended to the *afl*J as well as the *afl*J–*afl*R intergenic region, expression of *afl*R in the *sec*− strain was several fold lower than that observed in the *sec*+ strain, while *afl*J expression was barely detectable in the *sec*− strain. Western blot analysis indicated that despite AflR protein being present in the *sec*− strain, no toxin production resulted. Introduction of a second copy of *afl*R into the *sec*− strain increased *afl*R expression, but did not restore AF production. Lastly, reverse transcription-PCR analysis revealed that *lae*A was expressed in both *sec*+ and *sec*− strains. These results suggest that although *afl*R, *afl*J and *lae*A are necessary for AF production, they are not sufficient. We propose that the *afl*R and *afl*J expression may be regulated by element(s) downstream from *lae*A or from pathways not influenced by *lae*A.

Keywords: *Mycology, mycotoxins*

Introduction

Aflatoxins (AFs) are highly toxic and carcinogenic secondary metabolites produced mainly by three anamorphic species of the genus *Aspergillus*: *A. flavus*, *A. parasiticus* and *A. nomius* (Ehrlich et al. 2003). They are the most potent naturally occurring carcinogens known and have been linked to liver cancer and several other maladies in animals and humans (Validivia et al. 2001; Turner et al. 2003; Otim et al. 2005). Among the AF-producing fungi, the ubiquitous *A. flavus* and *A. parasiticus* are known pathogens of cotton, corn, peanuts and other oil-seed crops, producing toxin both in the field and during storage (Payne and Brown 1998). AF contamination continues to be a serious problem in many parts of the world (Richard and Payne 2003). In the USA alone, economic losses due to AF contamination of food and feed were estimated to be over US$500 million annually based mainly on market rejection and animal health impacts (Vardon et al. 2003). As a result, significant efforts are being directed towards developing strategies to control AF production or to prevent the survival of the fungi that produce AF.

Initially studied because of their negative impact on human and animal health (Eaton and Gallagher 1994), aflatoxins are perhaps the most well-known

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ISSN 0265–203X print/ISSN 1464–5122 online © 2007 Taylor & Francis
DOI: 10.1080/02652030701564563

class of mycotoxins, serving as a model system for the study of the genetics of mycotoxin biosynthesis and secondary metabolism in general (Cary et al. 2000; Bhatnagar et al. 2003; Yu et al. 2004a; Yu and Keller 2005). Our current understanding of the regulatory aspects of AF biosynthesis is a result of the combined efforts of several researchers in *A. flavus* and *A. parasiticus* or *A. nidulans* [sterigmatocystin (ST) producer, ST being a late AF precursor]. Two pathway-specific, divergently transcribed regulatory genes, *afl*R and *afl*J, have been shown to be necessary for the expression of all biosynthetic genes of the AF/ST pathways (Chang et al. 1993; Payne et al. 1993; Woloshuk et al. 1994; Brown et al. 1996; Meyers et al. 1998, and reviewed by Yu and Keller 2005). At the protein level, AflR and AflJ were shown to interact together (Chang 2003) and a lack of interaction between AflR and AflJ has been shown to contribute to the non-aflatoxigenicity in *A. sojae* (Chang 2004). However, unlike AflR, AflJ is not directly responsible for the turning on of the AF pathway genes; instead, it is proposed to act either as a transcriptional enhancer or as a co-activator of AflR (Meyers et al. 1998; Chang et al. 1999; Chang 2003). Some studies including the present study have suggested a possible link between fungal development and secondary metabolism (Kale et al. 1994, 1996; Guzman-de-Pena et al. 1998). Hicks et al. (1997) discovered that both asexual sporulation and ST production in *A. nidulans* require inactivation of the proliferative growth through inhibition of the FadA (G-protein) signalling pathway. Since then, additional factors in this G-protein/cAMP/protein kinase A pathway, their placement and their interactions have been identified (Shimizu and Keller 2001; Shimizu et al. 2003, reviewed by Yu and Keller 2005). In *A. parasiticus*, a similar signalling mechanism has been postulated to control AF production (Roze et al. 2004). Another gene of unknown function called *veA* was reported by Kato et al. (2003) and Calvo et al. (2004) as playing a role in the regulation of both AF/ST production as well as fungal development. A novel nuclear protein LaeA has been reported to be a global regulator of secondary metabolism in several aspergilli (Bok and Keller 2004). It was shown that deletion of *lae*A in *A. nidulans* resulted in the reduction of ST biosynthesis and other secondary metabolites including penicillin, while its over-expression yielded opposite results (Bok and Keller 2004; Bok et al. 2006).

Studies on AF regulation in our laboratory have utilized a group of highly stable, non-aflatoxigenic *A. parasiticus* morphological variants called *sec*− (for secondary metabolism negative; Kale et al. 1994). These variants were generated by serial transfer of non-sporulating mycelial macerates of the respective parental strain. The term 'secondary metabolism negative' was coined because thin-layer chromatography (TLC) analysis of these strains showed a lack of AF pathway intermediates along with undetectable AF production and a lack of other fluorescent or pigmented secondary metabolites made by the parental *sec*+ strains. Recent metabolic profile studies have shown that, indeed, the *sec*− strains display significantly reduced production of several secondary metabolites in addition to the AF pathway intermediates (Glassbrook et al. 2006) and efforts are underway to discern these secondary metabolites. Molecular analysis of the *sec*− strains showed that while their AF pathway genes were intact, the structural genes were not expressed at detectable levels. Further, the lack of conversion of exogenously fed precursors to AFs by the *sec*− forms suggested the absence of functional enzymes of the AF pathway in these strains (Kale et al. 1994, 1996). Additional studies revealed that the *afl*R gene was down-regulated in the *sec*− variants compared with their *sec*+ (for secondary metabolism positive) parents, although at the DNA level no sequence difference was found for *afl*R between the two strains (Kale et al. 2003). Northern blot and RT-PCR analysis revealed that the transcripts of *omtA* and *ver*-1, two structural genes of the AF pathway, were extremely low and unprocessed (introns not removed). In the same study, using parasexual crosses, despite increasing spore concentrations, the *sec*− strains failed to form heterokaryons and diploids with other *sec*− strains, while the heterokaryon test indicated that involvement of cytoplasmic elements such as mitochondria, plasmids or mycovirus in this phenomenon was unlikely (Kale et al. 2003). Reduced expression of *aflR* in the *sec*− strains at a level believed to be below the 'threshold' amount required for sufficient AflR protein production was proposed as a possible cause of this unusual phenotype (Kale et al. 2003).

In this study, an *A. parasiticus* SU-1 *sec*− variant was subjected to an array of molecular genetic analyses to determine if reduced expression of known regulators of AF production (i.e. AflR, AflJ, and LaeA) are responsible for the *sec*− phenotype.

Materials and methods

Fungal strains, media and culture conditions

The following fungal strains were used in this study. (1) *A. parasiticus* SU-1 (SRRC 143A), a wild-type strain previously used (Kale et al. 1996) for biochemical and genetic studies of both B group (B_1 and B_2) and G group (G_1 and G_2) aflatoxins. This strain was designated *sec*+ for secondary metabolism positive. (2) The progeny of

A. parasiticus SU-1 that no longer made any AF and designated SU-1 *sec*− (Kale et al. 2003). (3) *A. parasiticus* SU-1 (pHSP), the SU-1 strain harbouring a second copy of the *afl*R gene introduced by transformation with the plasmid pHSP (Chang et al. 1993). (4) A non-aflatoxigenic *A. flavus* NRRL 649 that contains a large deletion in the aflatoxin gene cluster (Woloshuk et al. 1995). (5) a niaD − mutant of the *A. parasiticus* SU-1 *sec*− strain was used as the recipient for transformation with pHSP, the plasmid harbouring a wild-type niaD gene and a second copy of the *afl*R gene (Chang et al. 1993). These strains, currently available at USDA, ARS, SRRC (New Orleans, LA, USA) are in the process of being deposited in the central culture collection. A *Saccharomyces cerevisiae* strain (InvSc1, Invitrogen) harbouring a plasmid expressing a haemagglutinin (HA)-tagged version of *aflR* (Cary et al. 2002) was used as a positive control in Western blot detection of AflR protein. Fungal cultures were maintained on potato dextrose agar (PDA, Difco, Detroit, MI, USA) plates. Fungal conidia were collected from PDA plates using a 0.5% Tween 80 solution. Fungal strains were grown at 29°C with constant shaking (150 rpm) for 64 h in YES liquid medium (2% yeast extract, 6% sucrose, pH 5.8) for isolation of nucleic acids and proteins. Mycelia were collected by vacuum filtration on 900-mm Whatman filter papers using a Buchner funnel, then flash frozen and ground to a fine powder using a pre-chilled mortar and pestle. All tissues were stored at −80°C until extracted. For TLC analysis, fungal strains were grown in defined liquid A&M medium (Adye and Mateles 1964).

DNA sequence analysis

The *aflJ* and *laeA* gene-coding regions and *afl*R/*afl*J intergenic region of both *sec*+ and *sec*− *A. parasiticus* isolates were PCR amplified, subcloned, and sequenced. Primers for *aflJ* and *aflJ*/*aflR* intergenic region were derived from the sequence of the *A. parasiticus* aflatoxin pathway gene cluster (GenBank accession no. AY371490). Primer pairs used were: 5′ *afl*J-CTTCAGATCACCTGTATTC TCAGCGAC, 3′ *afl*J-GCTGCAGCAAGAAAGG TCATTAGACT, generating a 1618 bp PCR product; 5′ *afl*R/*afl*J-GTCGCTGAGAATACAGG TGATCTGAAGAG, and 3′ *afl*R/*afl*J-CAACTCG TACAGCTATCCCGGAGCTTTCG, generating a 720 bp product. Approximately 500 ng of *A. parasiticus* SU-1 genomic DNA template were amplified using the PfuUltra Hotstart PCR 2 × Master Mix (Stratagene) in a 50 µl reaction mixture containing 100 pmol of each primer. Thermocycler parameters were as follows: one cycle of 95°C, 2 min; followed by 30 cycles of 95°C, 30 s; 63°C, 30 s; 72°C, 2 min; followed by a final extension at 72°C for 10 min. A total of 0.1 U of ExTaq polymerase was added to PCR reactions and incubated at 72°C for 10 min to add an adenosine residue to each end of the PCR products to facilitate subcloning into pCR 2.1-TOPO plasmid DNA (Invitrogen). Recombinant plasmids harbouring the correct PCR insert were sequenced using Beckman-Coulter CEQ8000 automated DNA sequencer (Beckman-Coulter, Fullerton, CA, USA). DNA sequence data were analysed using DNAMAN DNA analysis software (Lynnon Biosoft, Quebec, Canada). For sequencing of *lae*A, multiple PCR fragments were amplified using primers based on the genomic sequence of *A. flavus* (data not shown), using Platinum® PCR SuperMix (Invitrogen) as per the manufacturer's instructions. The resulting successful *laeA* primers and fragments were used for DNA sequencing on a Applied Biosystems, Inc. ABI 3730xl DNA Analyzer. The sequencing reactions were performed according to manufacturer protocols by terminator cycle sequencing with BigDye™ using 10% DMSO as a denaturant. The *A. parasiticus* SU-1 *lae*A coding sequences were submitted to the National Center for Biotechnology Information with accession numbers AY883018 (*sec*+) and AY883019 (*sec*−).

Nucleic acids isolation and analysis

For Southern hybridization analyses, fungal DNA was purified using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Total genomic DNA was subjected to exhaustive Hind III and KpnI double digestion, separated by 1% agarose gel electrophoresis, and vacuum transferred to Nytran Plus nylon membranes (Scheleicher and Schuell, Inc., Keen, NH, USA). Membrane prehybridization and hybridization was carried out in ULTRAhyb solution (Ambion, Austin, TX, USA). The membrane was hybridized with an α-^{32}P-dCTP labelled (Rediprime II Random Prime Labelling System, Amersham Biosciences, Piscataway, NJ, USA) DNA fragment corresponding to the *aflR* coding region. Membranes were hybridized overnight at 42°C followed by one wash at 42°C in 2 × SSC/0.1% SDS. An additional three washes at increasing temperatures of 42, 50, and 55°C were performed for 20 min each in 0.1 × SSC/0.1% SDS. The membranes were placed on Kodak X-OMAT AR autoradiography film (Eastman Kodak, Rochester, NY, USA) and allowed to expose for the desired time. Fungal total RNA was purified using the Qiagen RNeasy Plant Mini Kit (Qiagen) for Northern hybridization and quantitative RT-PCR analysis of *aflR* and *aflJ* expression. Total RNA was separated on a 2.2 M formaldehyde/1% agarose gel

and vacuum-transferred to Nytran Plus membranes. Membrane prehybridization and hybridization were carried out in ULTRAhyb solution. The membranes were hybridized with an α-^{32}P-dCTP labelled *aflR* fragment as described above or an approximate 1.4 kb PCR product representing the *aflJ* coding region. Membranes were hybridized overnight at 42°C then washed once with 2 × SSC/0.1% SDS at 42°C for 15 min followed by additional washes in 0.1 × SSC/0.1% SDS at 42°C and 50°C for 20 min each. The membranes were placed on Kodak X-OMAT AR autoradiography film (Eastman Kodak) and exposure allowed for the desired time.

Fungal transformation and PCR analysis

Aspergillus parasiticus protoplast generation and transformation was performed using the methods described by Ehrlich et al. (2004). Confirmation of the presence of pHSP integration into the fungal genome was performed by PCR. Total genomic DNA of putative SU-sec^{-} (pHSP) transformants was amplified using a primer specific for the PCR amplification of a region just inside the *aflR* coding region: 5′-TATCGATTACCTGCATCGAGA; and in the flanking pUC vector polylinker region of pHSP: 5′-CCAGTCACGACGTTGTAAAACG. The expected size of the PCR product was approximately 1.2 kb. Approximately 500 ng of genomic DNA template were added to a 50 µl reaction mix consisting of 1 × ExTaq buffer, dNTP mix, and 100 pmol of each primer, and 0.25 µl (1.25 U) of ExTaq Hot Start DNA polymerase (Takara Bio, Inc.). Thermocycler (iCycler Thermal Cycler, BioRad) parameters were as follows: one cycle of 94°C, 2 min; followed by 30 cycles of 94°C, 30 s; 55°C, 45 s; 72°C, 1 min; followed by a final extension at 72°C for 7 min.

Thin layer chromatography (TLC) analysis

For TLC analysis for AF production, strains were cultured in quadruplicate in 100 ml A&M liquid medium for 7 days at 150 rpm. The extraction procedure for secondary metabolites has been described in detail in Kale et al. (1996). The extracted dried samples containing the secondary metabolites were suspended in 1 ml of methylene dichloride and TLC was carried out by spotting 15 µl of each sample and known standards on prescored 250 µm-thick silica gel G plates (20 × 20 cm; Analtech). The plates were developed with ether:-methanol:water (96:3:1, by volume) for AF ($R_f = 0.44$ for AFB1). The plates were dried and viewed under long-wave ultraviolet light for fluorescent metabolites and digital photographs were taken.

Western blot analysis

For Western blot analysis, 0.5 g of frozen ground mycelia were combined with 500 µl of freshly prepared lysis buffer (10 ml of lysis buffer: one tablet of Roche Complete Protease Inhibitor Cocktail Tablets, 10 mM EDTA, 2 mM DTT, 0.5 mM phenylmethyl sulfonyl fluoride, 0.5 mM NaF, 0.1 mM Na_3VO_4, 500 µl of Sigma Protease Inhibitor Cocktail for use with fungal and yeast extracts, then equilibrated to 10 ml volume with Tris saline azide solution, 1 mM Tris base, 4 mM NaN_3, 0.14 M NaCl, pH 8.0) and homogenized using a mortar and pestle. The homogenate was transferred to a 2 ml microfuge tube and allowed to thaw on ice, then centrifuged for 6 min at 4°C at 14 000 rpm. After transferring the supernatant to a new microfuge tube an equal volume of 2 × sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.125 M Tris HCl) was added before heating at 100°C for 5 min. A total of 20 µg of crude extract was electrophoresed on 10% SDS-PAGE mini gel for 90 min at 100 V and subsequently transferred to a PVDF membrane (Scheleicher and Schuell, Keene NH, USA). Following transfer, the membrane was incubated in blocking solution (1% skim milk in TBST: 1 M Tris-HCl, 0.15 M NaCl, 0.1% Tween 80, pH 7.6) for 1 h at room temperature with gentle agitation. The blocked membrane was hybridized for 15 min with either anti-AflR antibody (1:3000 dilution in blocking solution, a gift from Professor John Linz, Michigan State University, MI, USA) or anti-Ver-1 antibody (1:500 dilution) for AflR and Ver-1 detection, respectively. Following incubation with the appropriate antibody, the membrane was washed 3 × with 25 ml of TBST for 5 min each followed by one wash in 25 ml sterile deionized water for 5 min. To visualize immunoreactive proteins, the membrane was incubated for 15 min in a 1:10 000 dilution of anti-rabbit IgG-horse radish peroxidase conjugate in blocking solution and washed 3 × with 25 ml of TBST for 5 min each followed by one wash in sterile deionized water for 5 min. The membrane was then incubated in 30 ml of Equilibrium substrate buffer (Lightshift Chemiluminescent EMSA Kit, Pierce, Rockford, IL, USA) for 5 min with gentle agitation. The membrane was blotted on a paper towel to remove excess buffer. In a clean tray, the membrane was uniformly covered in the substrate solution (1 ml of LightShift Luminol/Enhancer solution to 1 ml LightShift stable peroxide solution for 5 min without agitation. Excess substrate was removed from the membrane before exposure to near ultraviolet light for 1–10 s using a cooled CCD camera interfaced with a Fuji LAS-1000 Plus medical imaging system.

RT-PCR analysis

Total RNA was extracted from 24-, 36-, and 48-h YES samples using 100 mg of the fungal tissue and the TRIzol® Reagent (Invitrogen). The isolated RNA was DNase I (Invitrogen Corp., Carlsbad, CA, USA) treated following the manufacturer's protocols, with the addition of 0.33 µl of RNase OUT (Invitrogen Corp.) per µg of RNA. DNase-treated RNA was precipitated and treated a second time with DNase I. The primer pair used to amplify *laeA* by RT-PCR was 5′-TGGTACCGTAAT GGTTCTCC-3′ and 5′-ATATCAAGGCG ATCTTGCTC-3′. This primer pair was designed to amplify a 155-bp cDNA fragment and a 282-bp fragment from genomic DNA template. A 50 µl reaction mix containing 2 µg of total RNA, QIAGEN OneStep RT-PCR Enzyme Mix, QIAGEN OneStep RT-PCR buffer, dNTP mix, and 100 pmol of gene specific primers were used. Thermocycler (GeneAmp PCR System 9700, Applied Biosystems) parameters were as follows: 30 min at 50°C for cDNA synthesis, 15 min at 95°C for PCR activation, 45 cycles of 94°C, 45 s; 55°C, 45 s; and 72°C 1 in 30 s; followed by a final extension at 72°C for 10 min. The resulting PCR products were separated on 1% agarose gels to confirm the presence of the *lae*A gene.

Results and discussion

Aflatoxin contamination of food and feed products presents a significant threat to human and animal health. Several approaches can contribute to the reduction if not elimination of AF contamination. One such approach, which is the focus of our laboratory, is to understand the molecular and genetic basis of AF regulation so that the knowledge gained can be used in the development of novel strategies to control AF production in the field or under post-harvest storage conditions. This approach is also significant in that the fungus is the only common factor in toxin contamination of different crops.

In an effort to study regulation of AF production, we have generated a collection of *A. parasiticus sec* − strains by serial transfer of macerated mycelia using a wild-type, AF-producing SU-1 strain as well as spore colour and auxotrophic mutants that accumulated AF pathway intermediates as the *sec*+ parents (Kale et al. 1994). All *sec*− strains were highly stable, produced no AF or any pathway intermediates, and showed varying degrees of altered morphology and reduction in spore production compared with their *sec*+ parents (Kale et al. 1994). A lack of conversion of exogenously fed precursors to AFs by the *sec*− forms suggested an absence of functional enzymes of the AF pathway in these strains (Kale et al. 1996). DNA sequencing, Northern blot and RT-PCR analysis revealed that the *afl*R coding sequence was identical in the *sec*− and *sec*+ strains, still *afl*R expression was reduced and the transcripts of AF-pathway structural genes were extremely low and unprocessed (introns not spliced or incompletely spliced) in the *sec*− forms (Kale et al. 2003). A reduced, less than 'threshold', amount of *aflR* transcript and hence AflR protein required for activation of AF biosynthetic pathway genes was proposed as a probable cause of this unusual phenotype (Kale et al. 2003).

In this study the *afl*J and *afl*R–*afl*J intergenic regions were sequenced to identify any mutations at the nucleotide level that might explain the occurrence of the *sec*− phenotype. The *afl*R–*afl*J intergenic region contains, along with the promoter region of both *afl*R and *afl*J, putative binding sites for other AF-production related transcription factors including PacC (responsible for repressing transcription of acid-expressed genes under alkaline conditions; Tilburn et al. 1995), AreA (nitrogen regulatory protein; Chang et al. 1996) and BrlA (responsible for causing a switch from vegetative mycelial growth to conidiophore formation; Adams et al. 1988). Variability in the *afl*R–*afl*J intergenic region has been reported in five AF-producing Aspergilli and is believed to be associated with variable AF production among these strains (Ehrlich et al. 2003). However, in our study, the *afl*J and the *afl*R–*afl*J intergenic region was found to be 100% identical at the nucleotide level between the *sec*+ and the *sec*− isolates (data not shown) thus diminishing the possibility of an inactive AflR or AflJ due to mutations in the coding sequence or improper binding of the above-mentioned transcription factors as possible causes of the *sec*− phenotype. RT-PCR of RNA from *sec*+ and *sec*− strains showed that *afl*J was expressed in both.

Transformation with an extra copy of *afl*R (Chang et al. 1993, 1995; Ehrlich et al. 1998) or the carboxy-terminal transactivation domain region of *afl*R (Chang et al. 1999) has been shown to increase AF production substantially. Since *afl*R expression is noticeably lower in *sec*− compared with *sec*+ (Kale et al. 2003), the *sec*− strain was transformed with plasmid pHSP harbouring a copy of the *A. parasiticus afl*R gene and positive transformants were confirmed using Southern blot and PCR analyses (Figures 1 and 2). PCR analysis of selected transformants demonstrated the presence of an amplification product of about 1.2 kb for those transformants that harboured a second copy of aflR (Figure 1). Transformant T5 (Figure 1, lane 6) was chosen for subsequent analyses. Southern hybridization of HindIII-KpnI restriction digested genomic

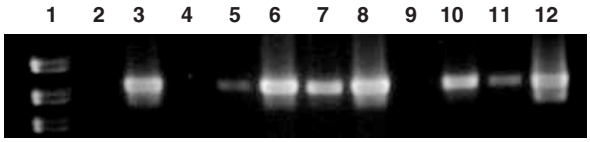

Figure 1. PCR analysis of *Aspergillus parasiticus* SU-1 *sec*− isolates transformed with plasmid pHSP. Lane 1, φX 174 Hae III DNA marker (Invitrogen); lanes 2–11, randomly selected *A. parasiticus* *sec*− (pHSP) transformants; lane 12, pHSP plasmid positive control. PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining.

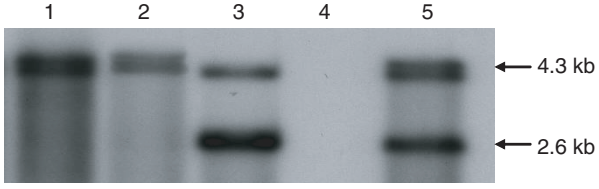

Figure 2. Southern hybridization analysis of *Aspergillus parasiticus* strains to verify the presence of a second copy of *afl*R in the *sec*− (pHSP) transformant T5 (lane 6 in Figure 1). The expected signals at 4.3 kb or at 4.3 and 2.6 kb for strains harbouring one or two copies of *afl*R, respectively, are denoted. Lane 1, *A. parasiticus* SU-1; lane 2, *A. parasiticus* SU-1 *sec*−; lane 3, *A. parasiticus* SU-1 (pHSP) containing an extra copy of *afl*R; lane 4, *A. flavus* NRRL 649; lane 5, *A. parasiticus* SU-1 *sec*− (pHSP), the *sec*− *afl*R transformant T5 (this study).

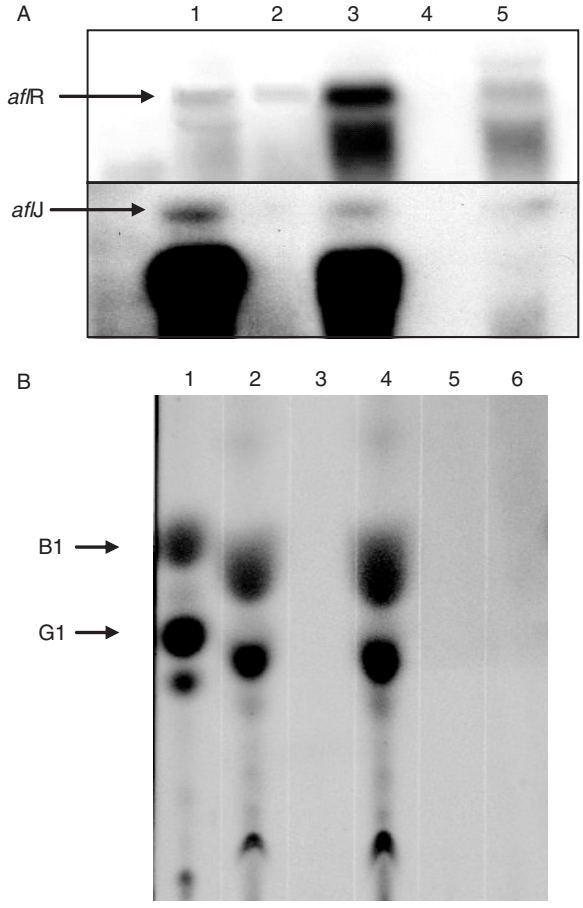

Figure 3. (A) Northern hybridization analysis of *afl*R and *afl*J transcripts. Lane 1, *A. parasiticus* SU-1; lane 2, *A. parasiticus* SU-1 *sec*−; lane 3, *A. parasiticus* SU-1 (pHSP); lane 4, *A. flavus* NRRL 649; lane 5, *A. parasiticus* SU-1 *sec*− (pHSP) transformant T5. The exact identity of the intense band below the *afl*J band is not known but is likely to be degraded form of the *afl*J transcript. It was noted in every repeated Northern blot analysis. (B) TLC analysis of *sec*+ and *sec*− strains. Lane 1, aflatoxin standard (Sigma); lane 2, *A. parasiticus* SU-1; lane 3, *A. parasiticus* SU-1 *sec*−; lane 4, *A. parasiticus* SU-1 (pHSP); lane 5, *A. flavus* NRRL 649; lane 6, *A. parasiticus* SU-1 *sec*− (pHSP) transformant T5. Note that migration on the TLC plate of aflatoxins B1 and G1 from the mycelial extracts was slightly less than that of the pure aflatoxin standard (lane 1).

DNAs demonstrated the expected hybridization signal for a single copy of *afl*R at 4.3 kb for SU-1 and SU-1 *sec*− and at 4.3 and 2.6 kb for the SU-1 (pHSP) transformant and SU-1 *sec*− (pHSP) transformant T5 that harboured a second copy of *afl*R (Figure 2). As expected, no hybridization signal was detected in the NRRL 649 aflatoxin cluster deletion mutant. Subsequent Northern blot analysis of transformant T5 total RNA indicated a higher level of expression of *afl*R and *afl*J transcripts compared with the untransformed *sec*− strain. No *afl*R or *afl*J transcripts were detected in the NRRL 649 mutant as expected. However, T5 still did not produce any detectable amounts of AF (Figure 3B). Western blots using AflR and Ver-1 antibodies were then conducted to determine any changes occurring in AflR protein levels between *sec*+ and *sec*− strains (preliminary results reported in Cary et al. 2006). Results demonstrated that the AflR protein was present in both the untransformed and the pHSP-transformed *sec*− strains while the Ver-1 protein was absent in both (Figure 4). The latter results were consistent with our earlier Northern hybridization studies in which transcripts of AF biosynthetic genes in the *sec*− strains were not detectable (only detected by RT-PCR in an unprocessed form) while *afl*R transcripts were lower but readily detectable in the *sec*− strain (Kale et al. 2003). Lastly, since the recently discovered L*ae*A protein has been shown to be involved in the production of an array of secondary metabolites (reviewed by Yu & Keller 2005), RT-PCR for this gene was performed (Figure 5). Results showed *lae*A expression in both the *sec*+ and *sec*− forms (Figure 5). The *lae*A levels were not significantly different between *sec*+ and *sec*− strains as shown by quantitative RT-PCR (data not shown), thus indicating that *lae*A is unlikely to be involved in causing the *sec*− phenotype. In another set of ongoing experiments, we have also performed RT-PCR analysis of *veA* and *brlA* to determine their possible role in the *sec*− phenomenon; and

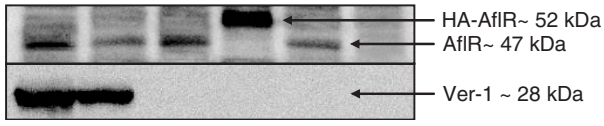

Figure 4. Western blot analysis of AflR and Ver-1 proteins from crude fungal protein extracts. Lane 1, *A. parasiticus* SU-1; lane 2, *A. parasiticus sec*−; lane 3, *A. parasiticus* SU-1 (pHSP), two copies of aflR; lane 4, HA-tagged AflR positive control from yeast; lane 5, *A. parasiticus sec*− (pHSP) transformant; lane 6, *A. flavus* NRRL 649 containing large deletion in AF cluster. The expected immunoreactive bands of 47 and 28 kDa for AflR and Ver-1 protein, respectively, are denoted by arrows. The HA-tagged version of AflR produced in the yeast strain had a mass of about 52 kDa.

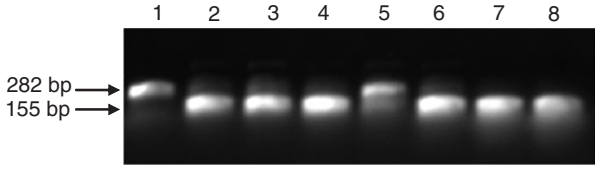

Figure 5. RT-PCR analysis of *laeA* transcripts. Total RNA was extracted from *A. parasiticus* SU-1 and SU-1 *sec*− strains grown in YES medium for 24, 36 and 48 h. The primers were designed to amplify a 155 bp *laeA* cDNA fragment or a 282 bp product from the genomic DNA template. The RT-PCR products were visualized by ethidium bromide staining. Lane 1, SU-1 genomic DNA control; lanes 2–4, SU-1 24-, 36- and 48-h RNA; lane 5, SU-1 *sec*− genomic DNA control; lanes 6–8, *sec*− 24-, 36- and 48-h RNA).

preliminary data (not shown) indicates expression of both genes in the *sec*+ and the *sec*− forms, thus suggesting that they too are unlikely to be responsible for the *sec*− phenotype.

In summary, based on our current knowledge of regulation of AF production in *A. parasiticus* (Cary et al. 2006), we have tested and ruled out three known AF-regulatory genes: *afl*R, *afl*J and *lae*A, as being responsible for loss of aflatoxigenicity in the *sec*− variants since the results suggest that the *sec*− phenotype is not caused by defects in *afl*R or *afl*J or *lae*A (at least at the DNA level). It is possible that the *sec*− strains lack additional positive regulators of secondary metabolic pathways that act independently of or in conjunction with AflR and AflJ. The *sec*− strains may also be overproducing AflR-binding repressor(s) of AF gene transcription similar to those reported by Chang et al. (1999). Displacement of such repressor(s) from AflR may be prevented due to absence or very low levels of AflJ in the *sec*− strains. Lastly, the results of Northern blots of the *sec*− (pHSP) transformant suggest that *afl*J transcription may not be completely under AflR control (Figure 5). Therefore, in addition to AflR, other as-yet-unknown factor(s) may regulate the expression of *afl*J in the *sec*− strains. Such a factor(s) may be a downstream component of pathways regulated by L*ae*A or may belong to pathways independent of L*ae*A involvement. The isogenic nature of the *sec*+/*sec*− pairs make them ideal candidates for further study of regulation of aflatoxin biosynthesis by microarray analyses. Recent advances in fungal genomics (Yu et al. 2004; Payne et al. 2006, OBrian et al. 2007, Wilkinson et al. 2007) in conjunction with recently initiated *A. flavus* genomic microarray studies will expedite the discovery of additional regulatory factors contributing to the formation of this intriguing class of secondary metabolism negative variants.

Acknowledgements

This research was funded by the NIH (MBRS) (Grant No. 5S06GM08008-31) to S. P. K. and by a cooperative research agreement between the US Department of Agriculture, Agricultural Research Service, Southern Regional Research Center, New Orleans, LA, and Tulane and Xavier universities. The assistance of Dr Ludmila Roze, Department of Food Science and Human Nutrition, Michigan State University, MI, in conducting the Western blot analysis is gratefully acknowledged. The work was also approved for publication as Journal Article No. J-11092 of the Mississippi Agricultural and Forestry Experiment Station, Mississippi State University. S. P. K. and J. W. C. contributed equally to this work.

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